PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 7/08, G01N 33/53 — A1

(11) International Publication Number: **WO 90/10644**

(43) International Publication Date: 20 September 1990 (20.09.90)

(21) International Application Number: PCT/SE90/00149

(22) International Filing Date: 8 March 1990 (08.03.90)

(30) Priority data:
8900899-9 14 March 1989 (14.03.89) SE

(71) Applicant *(for all designated States except US):* LOPE MEDICINE AB [SE/SE]; Dalagatan 35 A, S-113 23 Stockholm (SE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* OLSON, Lars [SE/SE]; Apeltunet 7, S-181 48 Lidingö (SE). PERSSON, Håkan [SE/SE]; Vreta Gård, S-147 00 Tumba (SE). EBENDAL, Ted [SE/SE]; Börjegatan 45 B, S-752 29 Uppsala (SE).

(74) Agents: HENNINGSSON, Gunnar et al.; Bergling & Sundbergh AB, Box 7645, S-103 94 Stockholm (SE).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.

Published
With international search report.

(54) Title: PEPTIDES AND ANTIBODIES FOR THE DETECTION OF NGF (NERVE GROWTH FACTOR) AND/OR PRECURSORS THEREOF

(57) Abstract

A peptide having the formula: Glu-Pro-His-Ser-Glu-Ser-Asn-Val-Pro-Ala-Gly-His-Thr-Ile, Gly-Asp-Lys-Thr-Thr-Ala-Thr-Asp-Ile-Lys-Gly-Lys-Glu, or functionally equivalent extended or truncated forms thereof excluding native NGF:s and precursors thereof; antibodies directed against such peptide; and a process for determining the presence of a mature NGF and/or a precursor thereof in a biological test specimen.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Fasso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic			SN	Senegal
CG	Congo	KR	Republic of Korea	SU	Soviet Union
CH	Switzerland	LI	Liechtenstein	TD	Chad
CM	Cameroon	LK	Sri Lanka	TG	Togo
DE	Germany, Federal Republic of	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

Peptides and antibodies for the detection of NGF (nerve growth factor) and/or precursors thereof.

The present invention relates to new peptides, to antibodies directed against said peptides and to diagnostic processes for determining the presence of mature nerve growth factors and precursors thereof.

Nerve growth factor, NGF, is a 118-amino acid protein that acts as a trophic factor for many sensory and sympathetic neurons in the peripheral nervous system and for cholinergic neurons in the brain. Also other non-cholinergic neurons in the central nervous system may be sensitive to NGF. It is therefore of interest to find diagnostic means to determine the presence and the level of NGF and precursors thereof to enable efficient treatment to alleviate different diseases of the nervous system, such as senile dementia of the Alzheimer type, Parkinson's disease, spinal cord injury, stroke, and developmental disturbances.

Accordingly, it is an object of the present invention to provide new peptides which can be efficiently used in such diagnostic procedures.

Another object of the invention is to provide antibodies against such new peptides for use in determining NGF levels using for example enzyme or radioimmuno assays to determine said levels.

Yet another object of the invention is to provide new processes for determining the presence of mature NGF:s and/or precursors thereof.

For these and other purposes the present invention provides for a peptide having the formula:

Glu-Pro-His-Ser-Glu-Ser-Asn-Val-Pro-Ala-Gly-His-Thr-Ile,

Gly-Asp-Lys-Thr-Thr-Ala-Thr-Asp-Ile-Lys-Gly-Lys-Glu.

One preferred peptide has the following formula: Glu-Pro-His-Ser-Glu-Ser-Asn-Val-Pro-Ala-Gly-His-Thr-Ile, said peptide corresponding to amino acid residues -103 to -90 in the human NGF precursor. The antibodies to this peptide

are effective in detecting the NGF precursor and can be used in immunological determination of the NGF precursor protein.

Another peptide according to the invention has the formula:

Gly-Asp-Lys-Thr-Thr-Ala-Thr-Asp-Ile-Lys-Gly-Lys-Glu, and this peptide corresponds to the conserved region of the NGF protein corresponding to amino acid residues 23 to 35 of the mature NGF protein. Tests performed in connection with the research leading to the present invention demonstrate recognition of the native NGF protein by antibodies raised to this peptide.

Relevant antibodies can be directed either against any of the peptides described above or both such peptides and native NGF:s. The antibodies may be either polyclonals or monoclonals.

The invention also provides for a process for determining the presence of mature NGF or a precursor thereof in a biological test specimen. Said process is based on the interaction between antibodies generated by immunization using a peptide according to the invention and said NGF or said precursor. Such determination may be qualitative or quantitative depending on the type of diagnosis performed and the object of the assay.

The test specimen subject to such assay may be constituted by mammalian brain tissue, such as brain tissue of human origin.

In the present disclosure, when reference is made to living animal body or living mammal body said terms are intended to include also man.

The present invention will be further illustrated below with reference to the appended drawings, wherein:

Fig. 1A shows homologous antibody binding to NGF peptides as a function of antiserum concentration;

Fig. 1B shows binding of affinity-purified peptide antibodies to their homologous peptides as a function of concentration of the immunoglobulines (Ig);

Fig. 1C shows antibody binding to different NGF-peptides shown as a function of antiserum concentration;

Fig. 2A shows peptide antibody binding to the β-NGF protein as a function of concentration of the affinity-purified immunoglobulines;

Fig. 2B shows comparison of binding to NGF peptide P3 in different antisera to β-NGF; and

Fig. 2C shows antibody binding to different synthetic NGF peptides by an antiserum to mouse β-NGF.

EXAMPLE 1

Synthesis of NGF Peptides

Hydrophilic areas suggested by the hydrophilicity values of Hopp TP, Woods KR (1981): Prediction of protein antigenic determinants from amino acid sequences. Proc.Natl.Acad. Sci. (USA) 78:3824-3828 in the pro-NGF and the mature NGF were selected for peptide synthesis (Table I). Peptides P1 to P6 were synthesized by Cambridge Research Biochemicals Ltd., England, at an estimated purity of greater than 80% and checked by amino-acid analysis, HPLC and mass spectrometry. Peptides P7 and P8 were kindly synthesized by Drs. Tamas Bartfai and Janis Abens at the Arrhenius Laboratory, Department of Biochemistry at the University of Stockholm, Sweden. Similarities to any other known protein sequences than NGF for each of these peptides were checked in the GeneBank without any extensive homologies found except to NGF.

EXAMPLE 2

Conjugation and Production of Peptide Antisera

All peptides were conjugated to keyhole limpet hemocyanin (KLH) before use in immunizations. KLH (8-10 mg) was in each case coupled with 3-10 mg of the peptide. Couplings were achieved with m-maleimidobenzoic acid N-hydroxy-succinimide or N-succinimidyl 3-(2-pyridyldithio)propionate to produce N-terminally bound conjugates of P1-P5 and P7-P8 and a C-terminally bound conjugate of P6. After dialysis the pep-

tide-KLH conjugates were used to immunize rabbits (Table II). Each rabbit received an initial dose of about 400 µg of the conjugate in Freund's complete adjuvant intramuscularly as well as via multiple intradermal injections. After 1 month, the rabbits received four weekly booster injections of about 200µg of the conjugate in incomplete adjuvant before being sacrificed.

Purification of Antibodies

Affinity columns were prepared for each of the eight peptides; 3 ml of ECH-Sepharose 4B (Pharmacia) was coupled with 2 mg peptide (coupling via a spacer arm to amino groups) using N-ethyl-N-(3-dimethylaminopropyl)-carbodiimide hydrochloride according to the manufacturer's instructions. Each gel was packed in a small column, and antiserum diluted 15--fold in 0.1 M Tris-HCl (pH 8) with 0.5 M NaCl was passed several times over the column. In most cases, the antiserum first had been taken over another column with an unrelated peptide to remove any possible nonspecifically binding components. After washing, the column was eluted with 4.5 M $MgCl_2$ in 0.05 M NaAc-buffer, pH 5.0, and the peak of eluting antibodies localized by the absorption at 280 nm. The peak was collected, dialysed extensively, and stored frozen.

Other Sera Tested

For comparison, normal rabbit antibodies were purified on protein A-Sepharose (rabbit no. 3). Antibodies to mouse β-NGF from rabbit (Ig 17) or sheep (Ig 28) were affinity-purified on NGF coupled to CNBr-activated Sepharose 4B as described earlier (Ebendal T, Olson L, Seiger A (1983): The level of nerve growth factor (NGF) as a function of innervation. Exp Cell Res 148:311-317; Ebendal T, Olson L, Seiger A, Belew M (1984): Nerve growth factors in chick and rat tissue. In Black IB (ed): "Cellular and Molecular Biology of Neuronal Development." New York: Plenum pp 231-242.) In addition, sera from another normal rabbit (no. 19) and from two rabbits im-

munized with mouse β-NGF (nos. 10 and 30) were examined in the present study. Finally, one rabbit (no. 46) receiving the first immunization with β-NGF (200 µg) and then four boosts with P3-KLH (200 µg each time) also was included.

EXAMPLE 4

Enzyme Immunoassay (ELISA)

Ninety-six-well immunoplates were coated with different peptides, KLH or β-NGF (all at 1 µg/ml) in 0.05 M carbonate buffer (pH 9.6). The plates were blocked with 1% bovine serum albumin (BSA), and then dilutions of the peptide antisera or the affinity-purified antibodies were added to the wells and left at 4°C overnight. After extensive washing, bound antibodies were detected with biotinylated antirabbit antibodies (Vector Labs., CA) followed by streptavidin-conjugated β-galactosidase (Bethesda Research Labs., MD). Enzyme activity was examined in a fluorometric plate reader (Dynatech Microfluor) after the addition of methylumbelliferyl-β--galactoside.

Bioassay of NGF-Inhibitory Activity

NGF activity was demonstrated in an assay with sympathetic chick embryo ganglia explanted to a collagen matrix (Ebendal T, Olson L, Seiger Å, Belew M (1984): Nerve growth factors in chick and rat tissue. In Black IB (ed): "Cellular and Molecular Biology of Neuronal Development." New York: Plenum pp 231-242). The influence of increasingly higher antiserum concentrations on NGF-induced fibre outgrowth was determined using this assay.

EXAMPLE 5

Immunohistochemistry

The NGF peptide antibodies also were characterized immunohistochemically in the salivary glands of adult male mice that had been kept in isolation at least 24 hr prior to sacrifice to ensure a resting state of the salivary glands.

The salivary glands were fixed by perfusion with 4% paraformaldehyde and further fixed by immersion in the same fixative for another 2 hr (Olson L, Ayer-LeLievre C, Ebendal T, Seiger A (1987): Nerve growth factor-like immunoreactivities in rodent salivary glands and testis. Cell Tissue Res 248:275--286). Cryostat section (5-14 µm) were incubated (4°C, overnight) with the affinity-purified peptide antibodies at concentrations 1-20 µg/ml in phosphate-buffered saline (PBS) with 0.3% Triton X-100. After washing, bound antibodies bound were visualized by incubation for 1 hr at room temperature with fluorescein-isothiocyanate-labelled antirabbit or antisheep antibodies. After further rinsing, sections were coverslipped in a mixture of glycerol and PBS (9:1), containing 0.1% p-phenylene diamine as an antifading agent (Johnson GD, de C Nogueira-Araujo GM (1981): A simple method of reducing the fading of immunofluorescence during microscopy. J.Immunol.Methods 43:349-350; Platt JL, Michael AF (1983); Retardation of fading and enhancement of intensity of immunofluorescence by p-phenylenediamine. J Histochem Bytochem 31:840--842). Sections were evaluated using epifluorescence microscopy (Nikon MicrophotFX) and the distribution and intensity of fluorescence estimated on a blind basis. Fluorescence intensities were semiquantitatively estimated on a 0-5 scale.

RESULTS

Antisera to the eight synthetic NGF peptides were raised in rabbits (Table I) after their conjugation to KLH. All peptide conjugates resulted in antisera that in ELISA tests bound to the appropriate NGF peptide (Table II). Serum titres (fold dilutions) in the ELISA ranged from 1:10,000 to 1:1,000,000. Peptide P2 gave high background values when used to coat the immunoplates and had to be conjugated with bovine serum albumin (BSA) before coating in order to achieve appropriate estimations of the titres of the antisera to P2. The titres measured with KLH-coated wells exceeded the titres for peptide binding and were in the range 1:1,000,000 to

1:10,000,000.

All peptide antisera were subjected to affinity chromatography on columns to which the peptides had been coupled. The recovery of immunoglobulins were in the range 50-200 µg per ml of antiserum (Table II). Recoveries corresponded only to some extent to the relative titres of the different antisera.

Binding to the appropriate NGF peptides by some of the peptide antisera as a function of antiserum dilution is shown in Figure 1A. The antisera bound to coats of the respective peptides at much higher rates than did normal rabbit serum at corresponding concentrations. Some peptide antisera performed better than others, although strict comparisons are not possible, as different peptides may adsorb differentially to the microwells.

Similar results were obtained with the affinity-purified peptide antibodies (Fig. 1B). The signal to KLH-coated immunoplates was at the same time reduced 50- to 500-fold (data not shown). Thus, it was concluded that the applied affinity purification scheme is useful to enhance the specific signal in, e.g., immunohistochemical studies.

The specificity of the peptide antibodies was further examined by ELISA, testing an antiserum raised to one peptide against coats of a range of the synthetic NGF peptides. An example is shown in Figure 1C, from which it is obvious that the homologous combination resulted in the best binding. However, peptides other than the one used for immunization were recognized to some extent (Fig. 1C).

These findings show that antibodies specific to the different synthetic NGF peptides resulted from immunizing the rabbits and that the antibodies could be efficiently purified by affinity chromatography. ELISA then was used to test whether or not any of the peptide antibodies recognized their corresponding peptide sequence in the native β-NGF protein. For comparison, rabbit antibodies (no. 17) to mouse β-NGF was included in the ELISA of NGF-coated immunoplates (Fig. 2A).

Of the peptide antibodies, only Ig 37 and 38, both against peptide P3, bound strongly to the mature β-NGF protein. The signal intensities obtained with these two antibodies were close to that of the affinity-purified, anti-NGF antibody (Fig. 2A). The other peptide antibodies bound NGF at least two orders of magnitude less efficiently, some not above the background levels obtained with normal rabbit immunoglobulin alone. Low levels of binding to native NGF also were found using the precursor-specific peptide antibodies. The findings suggest that peptide P3 is unique in generating antibodies that recognize the mature, native NGF protein.

Whether or not P3 represents an epitope of β-NGF that is found in polyclonal antisera raised to β-NGF also was investigated. Three antisera (AS 10, 17 and 30) were examined in a P3-ELISA, and all recognized the synthetic peptide well above background levels, although they differed in their relative ability to bind to P3 (Fig. 2B). The three NGFantisera bound P3 about one order of magnitude less efficiently than the anti-P3 antiserum AS 38 itself. An antiserum (AS 46) produced by immunization with β-NGF, followed by boosts with P3-KLH, gave the same high binding to P3 coats as the homologous P3 antiserum (Fig. 2B).

In order to check if any of the other peptides would be recognized by NGF antisera, AS 10, 17 and 30 were applied to the entire range of NGF peptides in an ELISA. The peptide P3 consistently resulted in high binding of antibodies (Fig. 2C). AS 17 also bound to P4, but this was less marked with AS 10 and 30. Antiserum AS 46 (the combined NGF/P3 antiserum) also recognized only P3 and none of the other peptides.

The possible interference with NGF activity by the peptide antibodies were studied in a bioassay using explanted sympathetic chick ganglia (Ebendal T, Olson L, Seiger A, Belew M (1984): Nerve growth factors in chick and rat tissue. In Black IB (ed):"Cellular and Molecular Biology of Neuronal Development." New York: Plenum pp 231-242). NGF-induced fibre outgrowths (stimulated by mouse β-NGF) were not blocked by

any of the peptide antisera, not even when present at high concentrations (3%) in the assays (data not shown). Affinity-purified antibodies to peptide P3 also failed to block the outgrowth response (tested at concentrations of up to 8 µg of Ig 38 per ml). This property of the peptide antibodies is reminiscent of the inability of monoclonal antibodies against NGF to block β-NGF-induced fibre responses in the ganglion assay. Furthermore, peptide P3 (at 100 ng/ml) could not substitute for NGF in neurite-outgrowth stimulation, nor did peptide P3 added in vast molar excess interfere with the biological activity of NGF. The NGF antiserum AS 46, at a thousand-fold dilution, was, however, blocking the sympathetic fibre outgrowth evoked by β-NGF.

Immunohistochemistry

The properties of the peptide antisera and affinity-purified antibodies also were examined using immunohistochemical staining of the male mouse submandibular gland. Various immunoglobulin preparations from the 19 rabbits immunized with the eight different peptides (Table II) were therefore tested immunohistochemically. In several cases, preadsorption tests with appropriate peptides were carried out. In addition, for peptides 4 and 5, the affinitypurified preparations were compared with the excluded fractions from the affinity--purification columns. Finally, comparisons were made to the distribution of immunofluorescence in the salivary gland using affinity-purified anti-NGF-antibodies.

EXAMPLE 6

The following peptide:
Glu-Pro-His-Ser-Glu-Ser-Asn-Val-Pro-Ala-Gly-His-Thr-Ile.
was synthesized by Cambridge Research Biochemicals Ltd., England, at an estimated purity of >80% and checked by amino acid analysis, HPLC and mass spectrometry. The peptide corresponds to position -103 to -90 of the human NGF precursor.

This peptide was subjected to the same treatments and procedures as described in Examples 2 to 5 above giving the same useful results. It was also noted that although the peptide corresponds to a sequence of the human NGF precursor it resulted in good binding of purified peptide antibodies also to NGF precursor of rat origin in spite of differencies in protein compositions.

The invention gives the important contribution to the assay techniques in that the levels of both NGF precursor and mature NGF per se can be determined using the peptides of the present invention. This is important in obtaining a correct diagnosis on nerve disorders since the relation between level of precursor and level of mature NGF may be decisive for determining the kind of treatment to be applied. Thus, abnormal levels of mature NGF in a human being can be either caused by overproduction thereof or by reduced consumption thereof. Therefore, the level of precursor can be decisive in establishing the nature of the disorder making it possible to find the correct treatment.

TABLE I

Synthetic NGF and Pro-NGF Peptides*

Peptide No	Species	Sequence	Position Start	Position End
P1	Chicken	(Tyr)-Glu-Thr-Lys-Cys-Arg-Asp-Pro-Arg-Pro-Val-Ser-Ser-Gly-Cys-Arg-Gly	54	69
P2	Chicken	Cys-Val-Leu-Ser-Arg-Lys-Ser-Gly-Arg-Pro	109	118
P3	Rat(mouse, chicken, human)	(Cys)-Gly-Asp-Lys-Thr-Thr-Ala-Thr-Asp-Ile-Lys-Gly-Lys-Glu	23	35
P4	Rat	Cys-Arg-Ala-Pro-Asn-Pro-Val-Glu-Ser-Gly	58	67
P5	Rat(mouse)	(Cys)-Glu-Pro-Tyr-Thr-Asp-Ser-Asn-Val-Pro-Glu-Gly-Asp-Ser-Val	-103	-90
P6	Rat(mouse, human)	Ser-Pro-Arg-Val-Leu-Phe-Ser-Thr-Gln-Pro-Pro-(Cys)	-38	-28
P7	Rat	(Lys)-Val-Leu-Ser-Arg-Lys-Ala-Ala-Arg-Arg-Gly	111	120
P8	Rat	(Cys)-Val-Lys-Ala-Leu-Thr-Thr-Asp-Asp-Lys-Gln-Ala	87	97

*Amino acid residues given within parentheses were added to the NGF sequence for conjugation purposes.

TABLE II

The NGF-Peptide Antisera Studied

Peptide No.	Rabbit AS No.	ELISA titre	Recovery of (µg/ml)*
P1	32	1: 200,000	60
	33	1: 100,000	90
	35	1: 10,000	15
	44	1:1,000,000	70
	48	1: 200,000	120
P2	34	1: 10,000	30
	36	1: 100,000	200
P3	37	1: 400,000	40
	38	1:1,000,000	80
P4	39	1:1,000,000	90
	40	1:1,000,000	100
P5	41	1: 100,000	60
	42	1: 10,000	30
P6	43	1: 100,000	80
	49	1: 100,000	100
P7	45	1: 10,000	150
	50	1: 20,000	90
P8	51	1: 200,000	220
	52	1: 100,000	40

*Values refer to recovery of specific peptide antibodies per ml antiserum by affinity chromatography.

CLAIMS

1. A peptide having the formula:
Glu-Pro-His-Ser-Glu-Ser-Asn-Val-Pro-Ala-Gly-His-Thr-Ile,

Gly-Asp-Lys-Thr-Thr-Ala-Thr-Asp-Ile-Lys-Gly-Lys-Glu,

or functionally equivalent extended or truncated forms thereof excluding native NGF:s and precursors thereof.

2. A peptide according to claim 1 having the formula:
Glu-Pro-His-Ser-Glu-Ser-Asn-Val-Pro-Ala-Gly-His-Thr-Ile.

3. A peptide according to claim 2 having the formula:
Gly-Asp-Lys-Thr-Thr-Ala-Thr-Asp-Ile-Lys-Gly-Lys-Glu.

4. A peptide acording to any preceding claim for diagnostic use in quantitative determination of NGF:s and precursors thereof in a living animal body.

5. Antibodies directed against the peptide of any preceding claim.

6. Antibodies according to claim 5 directed against the peptide of claim 2.

7. Antibodies according to claim 5 directed against the peptide of claim 3.

8. Antibodies directed against the peptide of any of claims 1 to 4 as well as against native NGF:s.

9. Antibodies according to any of claims 5 to 8 which are polyclonals.

10. Antibodies according to any of claims 5 to 8 which are monoclonals.

11. A process for determining the presence of a mature NGF and/or a precursor thereof in a biological test specimen using the interaction between the antibodies of any of claims 5 to 10 and said NGF or said precursor.

12. A process according to claim 11 for quantitative determination of a precursor of a mature NGF in a biological test specimen using the interaction between the antibodies of claim 6 , 9 or 10 and said precursor.

13. A process according to claim 11 for quantitative

determination of a precursor of a mature NGF in a biological test specimen using the interaction between the antibodies of claim 7, 9 or 10 and said NGF.

14. A process according to any of claims 11 to 13, wherein said test specimen is constituted by mammalian brain tissue.

FIG.1A

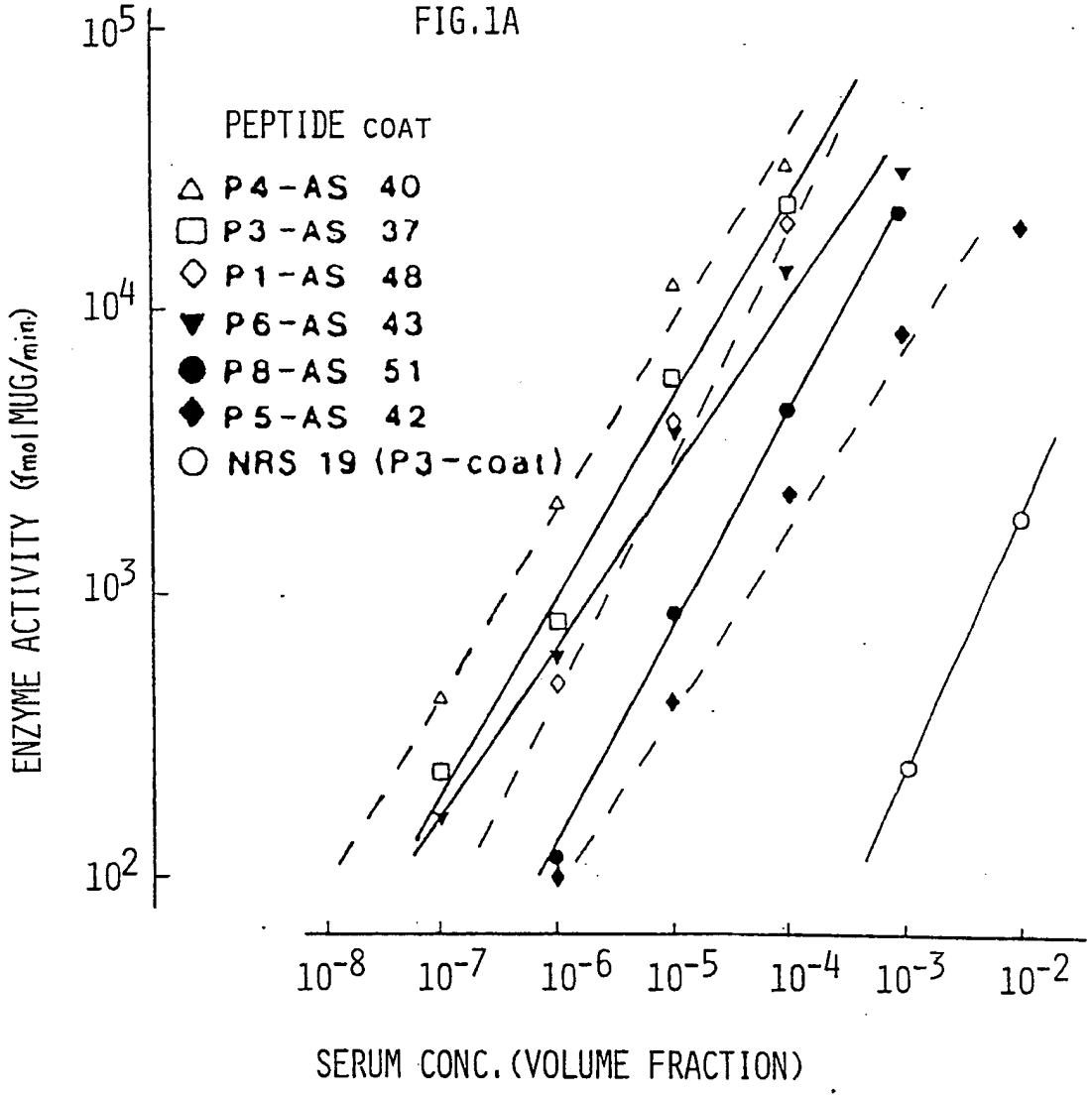

PEPTIDE COAT
P4-AS 40
P3-AS 37
P1-AS 48
P6-AS 43
P8-AS 51
P5-AS 42
NRS 19 (P3-coat)
ENZYME ACTIVITY (fmol MUG/min)
10^5
10^4
10^3
10^2
10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}
SERUM CONC. (VOLUME FRACTION)

FIG.1B

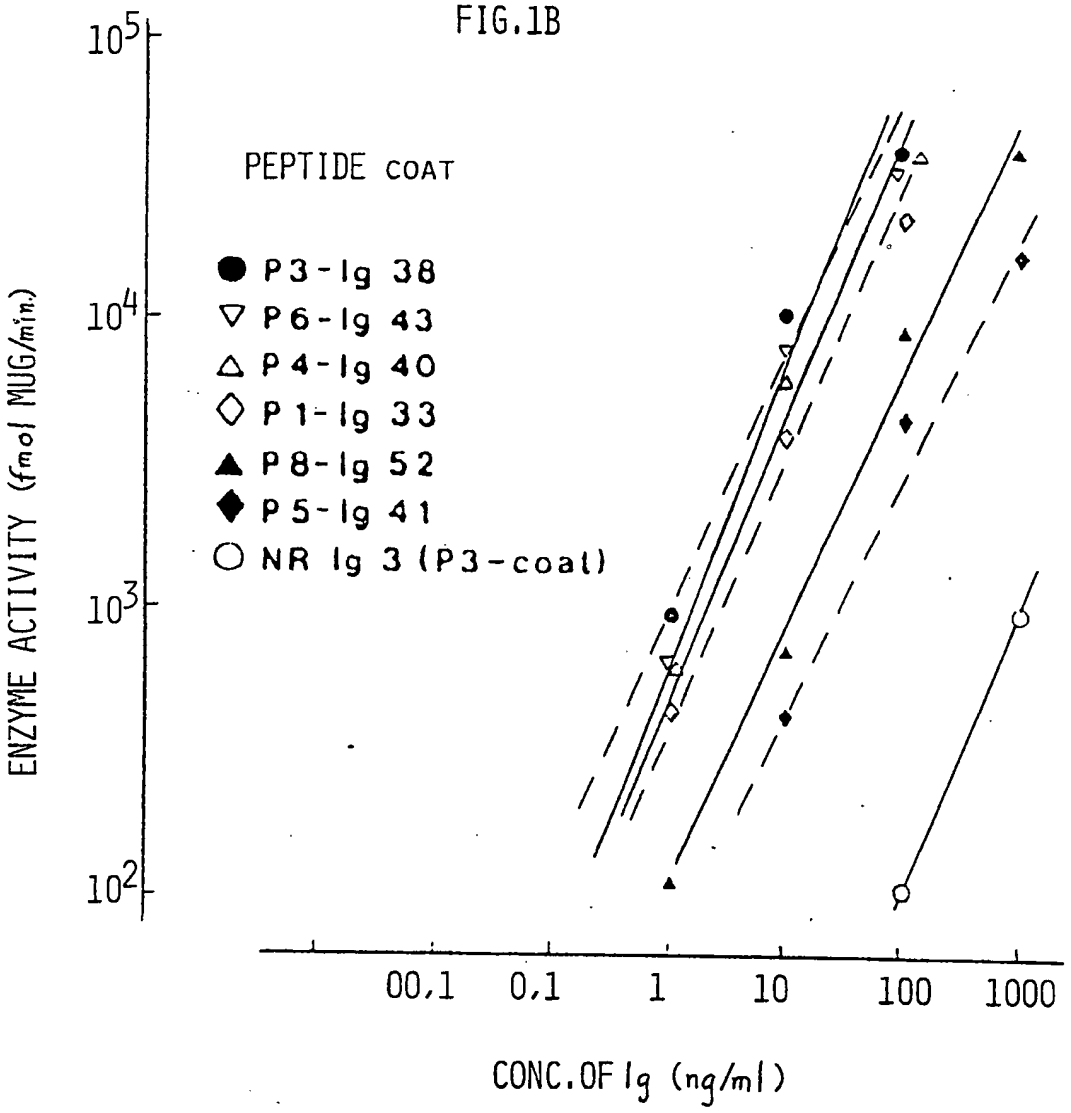

ENZYME ACTIVITY (fmol MUG/min.)
10^5
10^4
10^3
10^2
PEPTIDE COAT
P3-Ig 38
P6-Ig 43
P4-Ig 40
P1-Ig 33
P8-Ig 52
P5-Ig 41
NR Ig 3 (P3-coat)
00,1
0,1
1
10
100
1000
CONC. OF Ig (ng/ml)

FIG.1C

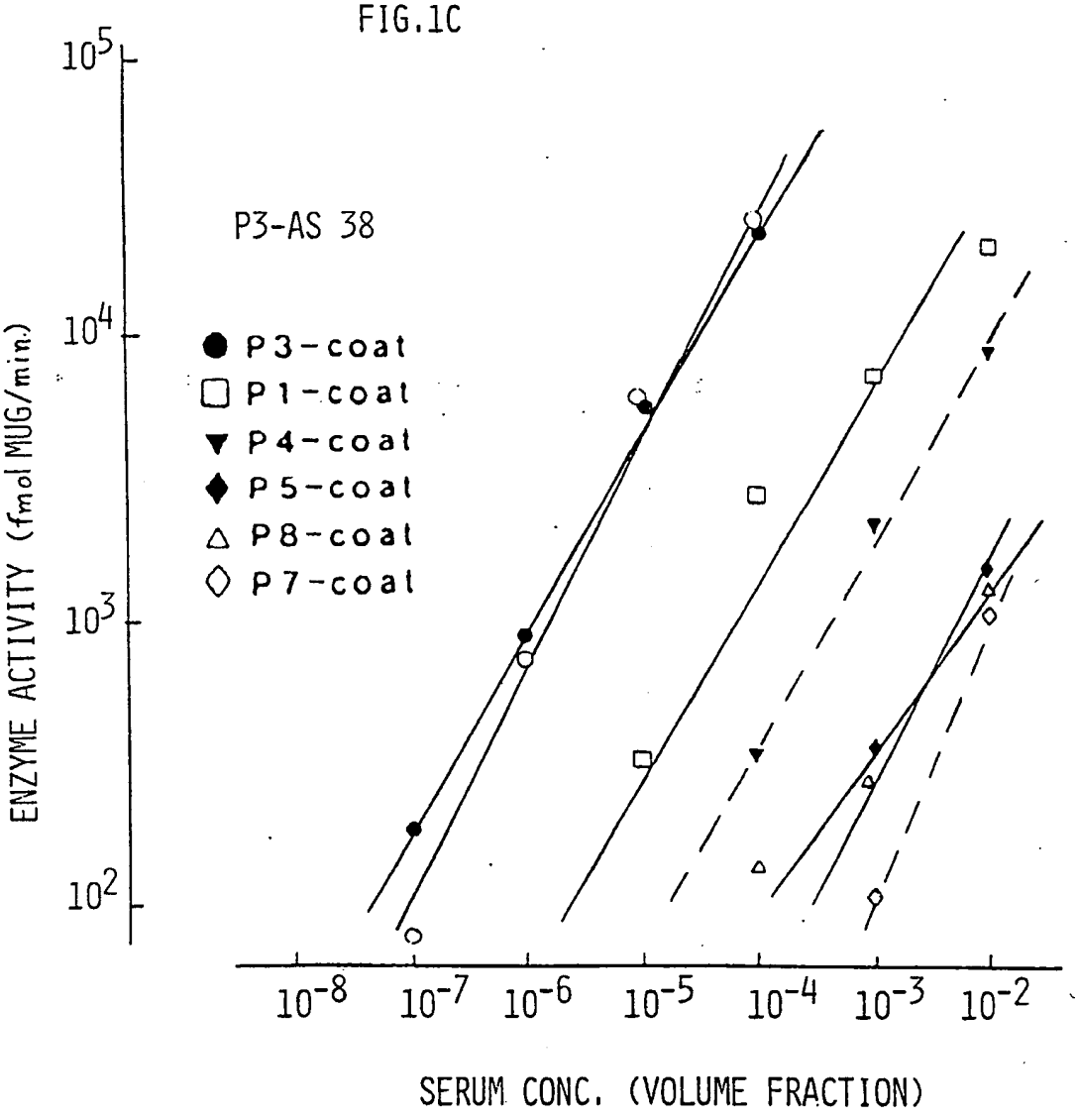

P3-AS 38
P3-coat
P1-coat
P4-coat
P5-coat
P8-coat
P7-coat
ENZYME ACTIVITY (fmol MUG/min)
10^5
10^4
10^3
10^2
10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}
SERUM CONC. (VOLUME FRACTION)

FIG.2A

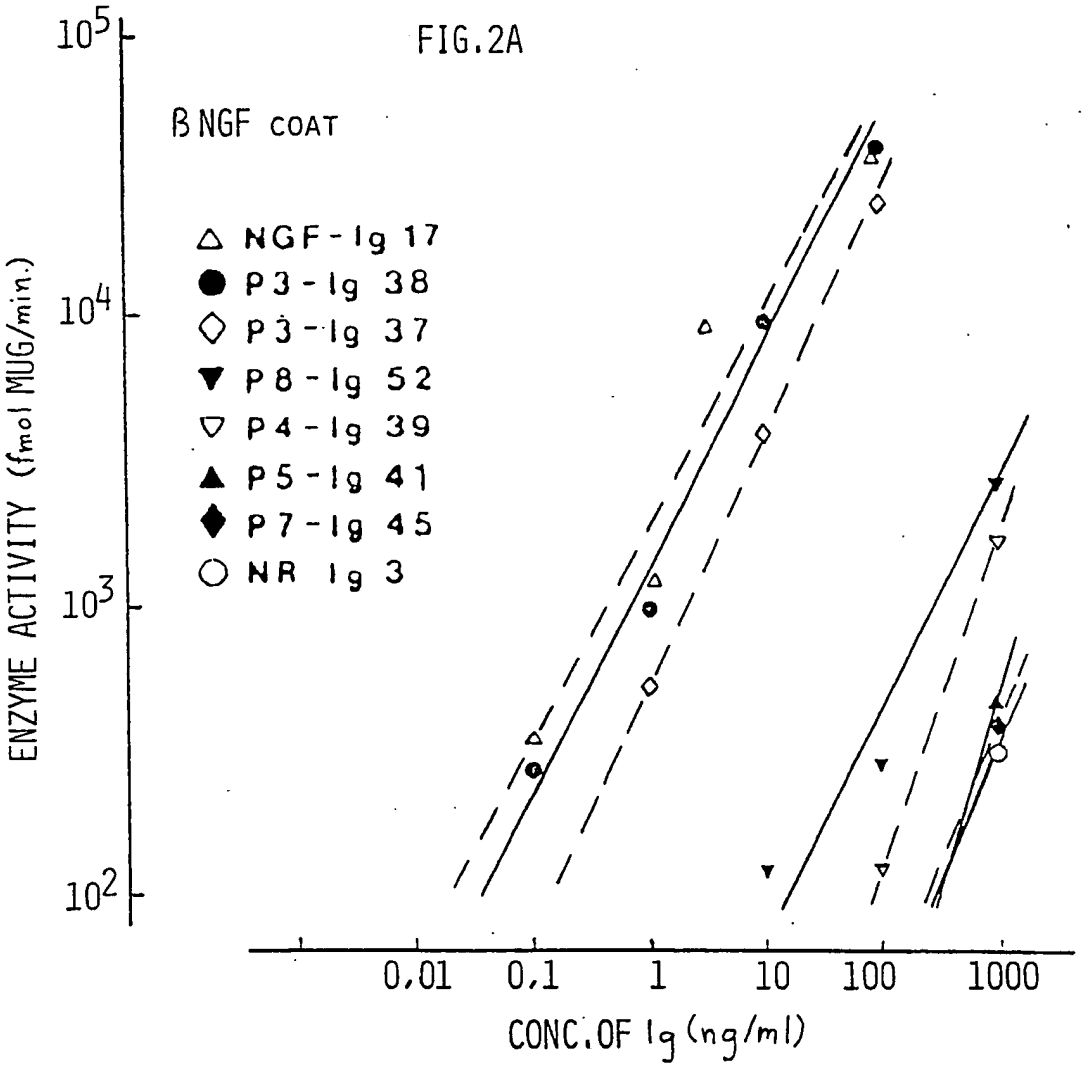

ß NGF COAT
△ NGF-Ig 17
● P3-Ig 38
◇ P3-Ig 37
▼ P8-Ig 52
▽ P4-Ig 39
▲ P5-Ig 41
◆ P7-Ig 45
○ NR Ig 3
ENZYME ACTIVITY (fmol MUG/min.)
10^5
10^4
10^3
10^2
0,01
0,1
1
10
100
1000
CONC. OF Ig (ng/ml)

FIG.2B

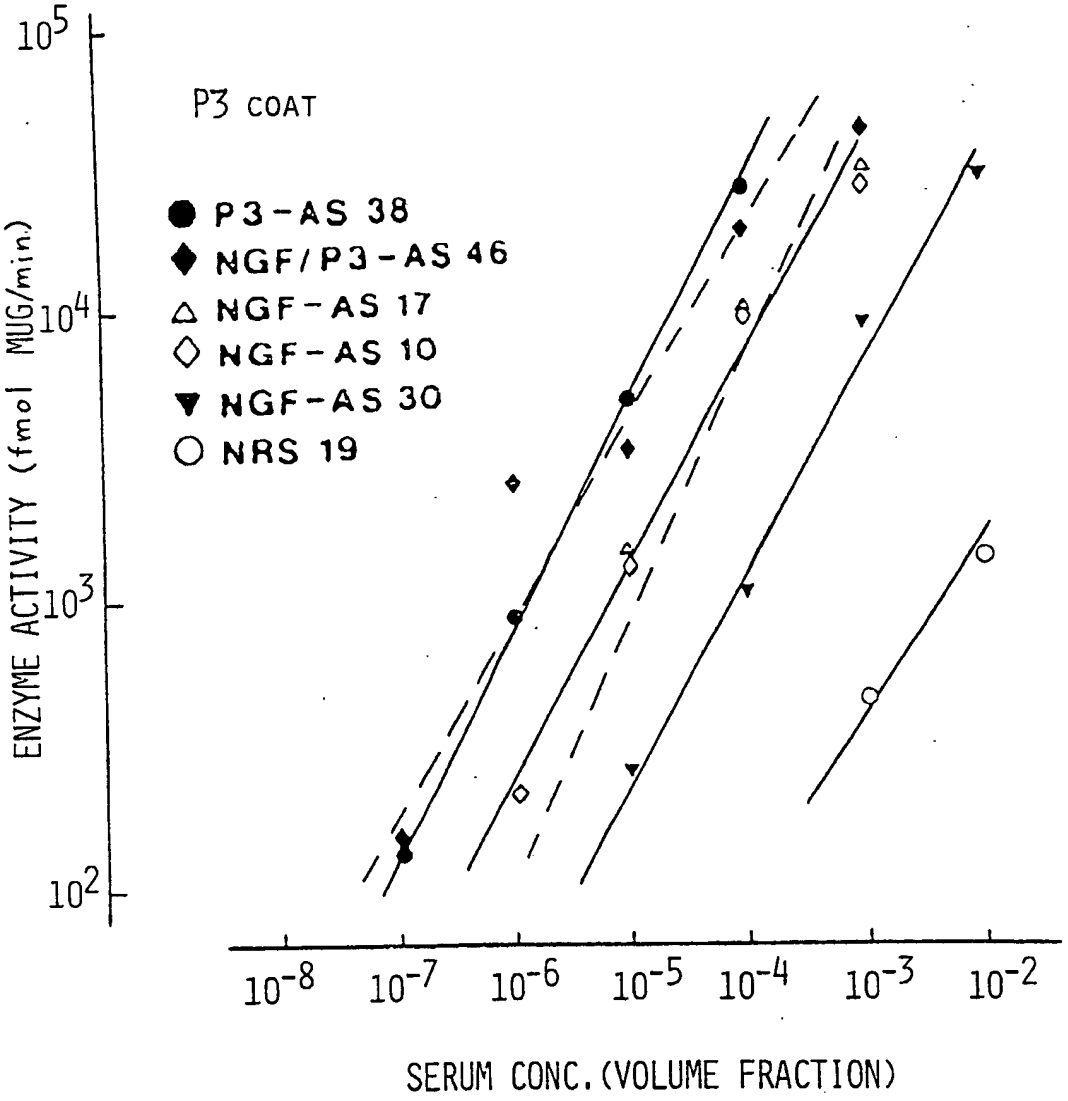
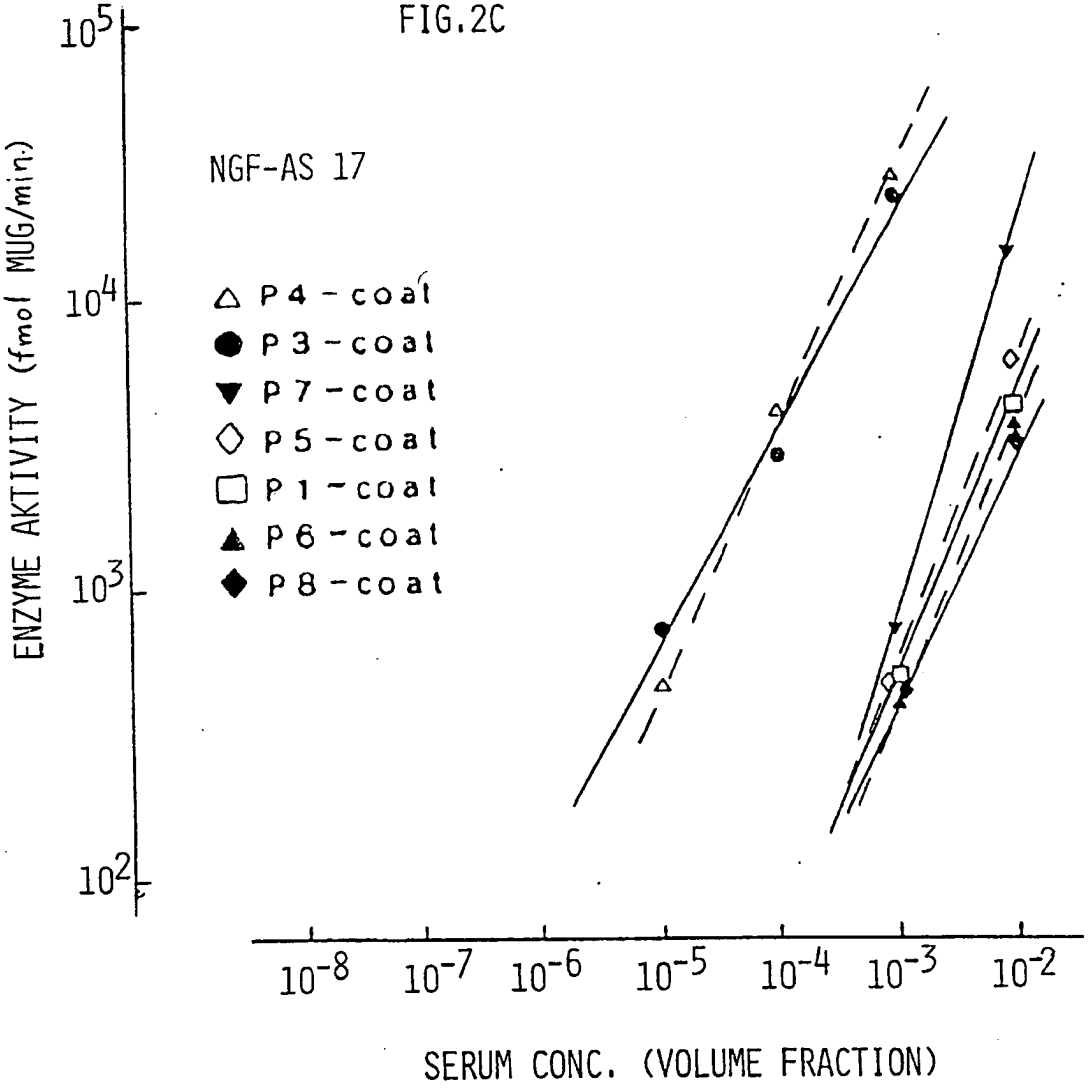

P3 COAT
P3-AS 38
NGF/P3-AS 46
NGF-AS 17
NGF-AS 10
NGF-AS 30
NRS 19
ENZYME ACTIVITY (fmol MUG/min.)
10^5
10^4
10^3
10^2
10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}
SERUM CONC.(VOLUME FRACTION)

FIG.2C


NGF-AS 17
P 4 - coat
P 3 - coat
P 7 - coat
P 5 - coat
P 1 - coat
P 6 - coat
P 8 - coat
ENZYME AKTIVITY (fmol MUG/min.)
10^5
10^4
10^3
10^2
10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}
SERUM CONC. (VOLUME FRACTION)

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 90/00149

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 7/08, G 01 N 33/53

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	METHODS IN ENZYMOLOGY, Vol. 147, 1987 Sigrun Korsching et al: "Two-Site Enzyme Immunoassay for Nerve Growth Factor ", see page 167 - page ff	8,9
Y	--	1-7,10-14
Y	THE EMBO JOURNAL, Vol. 5, No. 7, 1986 R. Meier et al: "Molecular cloning of bovine and chick nerve growth factor (NGF): delineation of conserved and unconserved domains and their relationship to the biological activity and antigenicity of NGF ", see page 1489 - page 1493 see in particular fig 3 and pages 1491-2	1-14
	--	

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13th June 1990	1990 -06- 20
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carl-Olof Gustafsson

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	JOURNAL OF NEUROSCIENCE RESEARCH, Vol. 22, 1989 E. Dicou et al: "Synthesis of Chimeric Mouse Nerve Growth Factor Precursor and Human B-Nerve Growth Factor in Escherichia coli: Immunological Properties ", see page 13 - page 19 see in particular page 18 --	1-14
Y	THE EMBO JOURNAL, Vol. 5, No. 7, 1986 Ted Ebendal et al: "Structure and expression of the chicken B nerve growth factor gene ", see page 1483 - page 1487 see in particular fig 3 and page 1486 --	1-14
X	METHODS IN ENZYMOLOGY, Vol. 147, 1987 Kenneth E. Neet et al: "Derivation of Monoclonal Antibody to Nerve Growth Factor ", see page 186 - page ff --	8,10
P,X	JOURNAL OF NEUROSCIENCE RESEARCH, Vol. 22, No. 3, 1989 T. Ebendal et al: "Characterization of Antibodies to Synthetic Nerve Growth Factor (NGF) and ProNGF Peptides ", see page 223 - page 240 --	1-14
P,X	Chemical Abstracts, volume 111, no. 23, 4 December 1989, (Columbus, Ohio, US), Dicou, Eleni : "Interaction of antibodies to synthetic peptides of proNGF with in vitro synthesized NGF precursors ", see page 125, abstract 209702k, & FEBS Lett 1989, 255(2), 215- 218K --	1,2,6,8-10
P,X	WO, A1, 8909225 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 5 October 1989, see pages 14-22 and claims --	1,3

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/SE 90/00149

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 90-05-07
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 8909225	89-10-05	AU-D-	3418389	89-10-16
		EP-A-	0335637	89-10-04